Back to the future: museum specimens in population genetics

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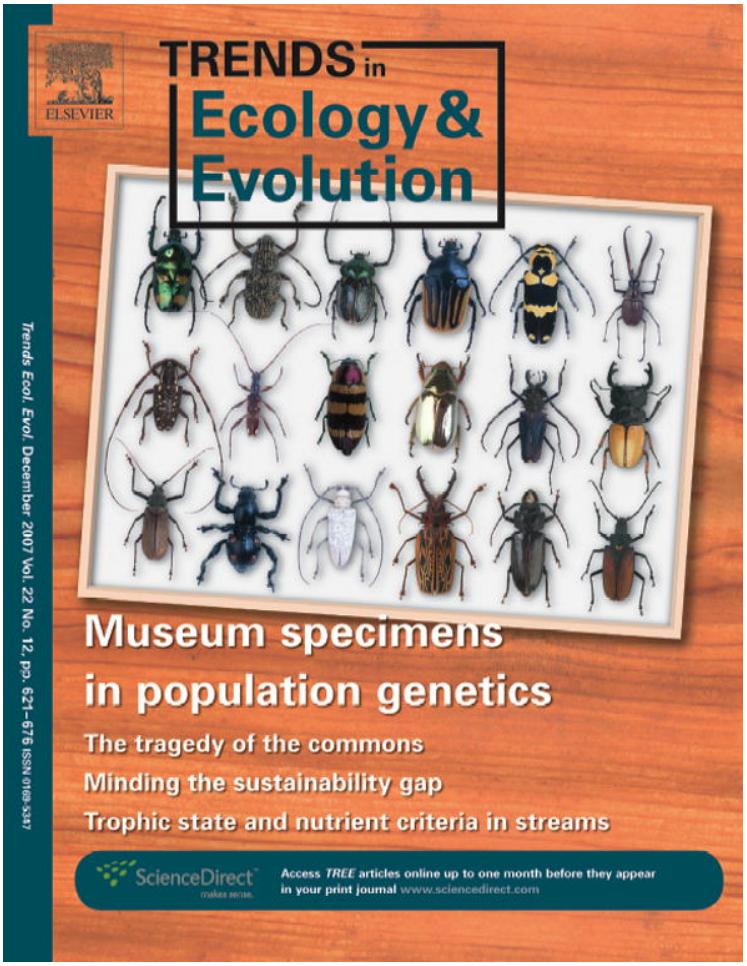

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Back to the future: museum specimens in population genetics

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Museums and other natural history collections (NHC) worldwide house millions of specimens. With the advent of molecular genetic approaches these collections have become the source of many fascinating population studies in conservation genetics that contrast historical with present-day genetic diversity. Recent developments in molecular genetics and genomics and the associated statistical tools have opened up the further possibility of studying evolutionary change directly. As we discuss here, we believe that NHC specimens provide a largely underutilized resource for such investigations. However, because DNA extracted from NHC samples is degraded, analyses of such samples are technically demanding and many potential pitfalls exist. Thus, we propose a set of guidelines that outline the steps necessary to begin genetic investigations using specimens from NHC.

Introduction

Given that evolution is change over time, documenting and understanding temporal patterns has long been at the heart of evolutionary studies. In disciplines such as palaeontology, inferences about evolutionary processes are drawn from the analyses of temporal patterns in the fossil record. Similarly, our understanding of microevolutionary processes (i.e. changes in gene frequencies over time) has often involved the analyses of records taken over several years; Dobzhanksy's [1] early studies of microevolution among *Drosophila* used this approach, a tradition that continues among students of this model organism today [2]. However, such microevolutionary studies were often limited to certain taxa and questions because the time available to document temporal changes was often limited to a few generations.

How can these limitations be overcome? Long term studies, running over several decades, are one possibility and they are yielding fascinating insights, for example, into the role of reinforcement and character displacement in adaptive radiation and speciation [3,4]. Another approach, which gives longer time series, is to extend the data back in time using well preserved fossil samples or specimens from natural history collections (NHC). Here, we review the use of specimens from NHC for the study of evolutionary change. We aim to increase awareness of both the methodological limitations involved in using molecular methods with NHC specimens and their future potential.

We focus on studies of evolutionary change rather than the widespread use of NHC specimens in phylogenetics and phylogeography (e.g. Ref [5]) or pathogen origin and dynamics (e.g. Ref [6]). Similarly, we limit ourselves to studies of NHC specimens and do not consider studies of ancient DNA (Box 1; see [7–8] for excellent reviews on the latter). Although ancient DNA studies have yielded spectacular results [9,10], they will remain restricted to a relatively small set of species because the samples required for such work are rare and difficult to obtain. By contrast, NHC specimens generally cover a broader taxonomic range and are more easily obtained, thus enabling a wider range of questions and taxa to be studied.

NHC samples in conservation genetics

A large proportion of empirical studies of NHC samples published to date contrast past and recent genetic diversity in threatened and endangered populations or species (Table 1). Many now endangered or extinct populations and species became so within the past two centuries [11], a time period that coincides with the establishment of the majority of NHC (but see Ref [12]). As a result, specimens stored in NHC often represent the genetic diversity of populations shortly before significant anthropogenic influence. By inferring temporal changes in neutral genetic diversity, biologists can obtain estimates of the magnitude of anthropogenic influences on population sizes and connectivity (i.e. gene flow between populations) [13] and they can detect cryptic introductions or genetic introgression. Such insights could guide future conservation action.

Population declines and loss of genetic diversity

Low genetic variation in endangered populations is of conservation concern because genetic variation is the raw material required for future adaptive evolution. Low levels of genetic variation can be the consequence of recent population declines, or it can represent an ancestral state. Differentiating these two causes is therefore an important task in conservation biology and it can be achieved by comparing levels of genetic variability among NHC samples collected before a genetic bottleneck with those found in current populations.

One of the earliest studies to use such an approach was an investigation of an endangered population of the greater prairie chicken (*Tympanuchus cupido*) [14]. This study provided direct evidence for a human-induced decrease in genetic diversity over time: specific alleles present in the NHC samples were no longer present in the current population. Such 'ghost alleles' have subsequently

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Box 1. Ancient DNA versus DNA from NHC

NHC specimens have often been included in the definition of ancient DNA [7]. However, there are several differences between ancient DNA and that extracted from NHC. For example, ascertaining authenticity is crucial in ancient DNA work whereas in NHC-based studies authenticity is more easily ascertained by comparisons with results from high-quality DNA samples taken from extant populations. We therefore propose to make a distinction based on the criteria in Table I, even if there is not always a sharp contrast. Based on our criteria, working with DNA from NHC specimens is feasible when dedicated laboratory facilities are available for working with low-quality DNA and when the guidelines outlined in Boxes 3 and 4 can be fulfilled. By contrast, working with ancient DNA is methodologically considerably more demanding and, therefore, remains the domain of specialist groups and laboratories.

Table I. Differences between DNA extracted from NHC and ancient samples

	DNA from NHC	Ancient DNA
Sample origin	Museum, private and archive collections, herbarium	Archaeological and palaeontological sites, museum
Sample age	≤200 years	~100 000 years[a]
Sample size	Often large enough for population sample	Generally small[b]
Tissue types	Several (see Box 2)	Mainly hard tissue
Hominid samples	Seldom	Common
Main causes of DNA decay	Preservation methods, storage condition	Physical factors at sampling site, storage conditions
Level of DNA decay	High	Very high[c]
Variance of DNA quality among samples	Very high	High
Microsatellite amplification	Common	Seldom
Problems with authenticity	Moderate	Very high
Risk of contamination	High	Very high

[a]Sample age of one million years might be possible.
[b]The number of investigations with larger sample size is currently increasing.
[c]Although some ancient samples can yield higher DNA quality.

been reported in several bottlenecked animal populations. By contrast, other studies have reported stable genetic diversity despite declines in population size [24–26]. For example, no obvious loss of genetic diversity was detected among Canadian peregrine falcons (*Falco peregrinus*) despite a DDT induced bottleneck [25].

Inferring the genetically effective population size

The genetically effective population size (N_e) is a key parameter that determines the rate of loss of genetic variation owing to genetic drift. Measuring N_e is thus of interest in conservation biology, but is notoriously difficult [27]. However, several methods based on temporal samples have been developed, including forward probabilistic approaches and backwards coalescence approaches (reviewed in Ref [27]). Using microsatellites amplified from DNA extracted from historical samples, temporal estimates of N_e have been obtained in several different fish species [16,28–30], grizzly bears (*Ursus arctos*) [31], greater prairie chicken [14] and leopard frog (*Rana pipiens*) [32]. Some of these N_e estimates were lower than anticipated whereas others were higher. This pattern highlights the fact that including NHC samples in such analyses can prevent us from reaching misleading conclusions.

Changes in connectivity

Habitat fragmentation often leads to changes in population connectivity. With increasing population fragmentation, migration among populations and, thus, gene flow is expected to decrease. This leads to a reduction in genetic diversity within populations and an elevation of genetic differentiation among populations [18,33]. Such an increase in spatial genetic differentiation over a time period of six to seven generations has been reported for the endangered Spanish imperial eagle (*Aquila adalberti*) [33]. This finding suggests that future management policies should attempt to restore the ancestral panmictic situation through habitat restoration or translocations.

Detecting introductions and introgression

Specimens from NHC have been used also to detect new introductions and to assess the rate of genetic introgression into indigenous populations. For example, the cryptic invasion of a non-native genotype of the common reed (*Phragmites australis*) in North America during the past century was detected by sequencing samples collected worldwide at two noncoding regions of the chloroplast [34]. Similarly, archival fish scales have been used to quantify the genetic consequences for wild fish populations of stocking or of aquaculture escapees [35,36]. Stocking of Spanish Atlantic salmon (*Salmo salar*) since the 1970s appears to have increased the mitochondrial DNA diversity of four endangered populations. However, it failed to halt the ongoing population decline [35].

NHC samples in evolutionary biology

Fisher and Ford [37] provided an early example of the use of NHC specimens to study evolutionary change directly. Their 1947 study of the spread of the *medionigra* gene among the scarlet tiger moth (*Callimorpha dominula*) provided clear evidence for gene frequency change owing to natural selection. Since then, surprisingly few studies have taken advantage of the evolutionary history preserved in NHC samples to investigate the molecular footprint of selection. One such study investigated the evolutionary genetic mechanisms that underlie the rapid evolution of insecticide resistance in the blowfly (*Lucilia cuprina*) in Australia [38]. Sequence data from 16 pinned NHC specimens collected before the first use of organophosphate insecticides revealed that the rapid evolution of insecticide resistance was because of the pre-existence of mutant alleles in the historical gene pool and that the

Table 1. A selection of studies contrasting past and recent genetic diversity using specimens from NHC[a,b]

Species	Marker[c]	Time span[d]	Sample[e]	N[f]	Summary	Refs
Plants						
Common reed *Phragmites australis*	1400 bp cpDNA	Before 1910; after 1960	5/2	62/283	Cryptic invasion of non-native genotype	[34]
Marsh orchid *Anacamptis palustris*	250–280 bp plastid DNA	1832–recent	5/4	89/316	Genetic variation in time and space	[72]
Insects						
Sheep blowfly *Lucilia cuprina/sericata*	287 bp *LcαE7*	1930–1990	-/2	16/35	Evolution of insecticide resistance	[38]
Adonis blue *Polyommatus bellargus*	4	1897–1999	1/2	20/74	High genetic drift in an insect population	[73]
Fish						
Northern pike *Esox lucius*	7	1961–1993	1/3	196/72	Temporal N_e estimation of introduced population	[29]
Atlantic salmon *Salmo salar*	6	1913–1994	7/4	228/90	Genetic diversity between and within extant and extinct populations	[74]
Newfoundland cod *Gadus morhua*	6	1964–1994	5/3	574/570	Stability of genetic structure despite population size decline	[24]
Brown trout *Salmo trutta*	8	1910–1992	5/3	191/311	Estimation of N_e and temporal stability of genetic structure	[28]
	9	1945–2000	2/7	146/397	Genetic effects of stocking domestic trout into wild populations	[36]
New Zealand snapper *Pagrus auratus*	7	1950–1998	2/5	372/96	Genetic diversity and low N_e in two overexploited populations	[16]
Steelhead trout *Oncorhynchus mykiss*	7	1958–1998	3/4	180/90	Temporal diversity and N_e for three populations	[30]
Brown trout *S. trutta*	6; 1 *Satr-UBA*[g]	1958–1995	1/5	232/50	Temporal variation of MHC class I gene during aquaculture activities	[68]
Atlantic salmon *S. salar*	1409 bp RFLP	1948–2002	4/5	592/125	Effects of stocking on mitochondrial diversity	[35]
Amphibians						
Leopard frog *Rana pipiens*	7	1971–2001	5/2	204/188	N_e and temporal stability of genetic structure	[32]
Birds						
Greater prairie chicken *Tympanuchus cupido*	6	1930s–recent	4/2	15/127	Genetic diversity of pre- and post-bottleneck populations	[14]
Mauritius kestrel *Falco punctatus*	12	1830–recent	7/2	52/250	Genetic monitoring of a pesticide-induced bottleneck	[17]
Greater prairie chicken *Tympanuchus cupido*	384 bp; 6	1951-2000	4/2	125/81	N_e and temporal genetic variation in bottlenecked populations	[18]
White-headed duck *Oxyura leucocephala*	192 bp	1861–2003	-/2	67/46	Loss of genetic diversity	[21]
Peregrine falcon *Falco peregrinus*	405 bp; 11	1885–2004	-/2	95/184	Pesticide-induced bottleneck in Canadian falcons	[25]
Spanish imperial eagle *Aquila adalberti*	345 bp; 10	1860-recent	1/2	34/79	Effects of fragmentation on spatiotemporal genetic structure	[33]
Mammals						
Kangaroo rat *Dipodomys panamintinus*	225 bp	1911–1988	3/2	49/63	Continuity of spatial and temporal mtDNA diversity	[75]
Northern hairy-nosed wombat *Lasiorhinus krefftii*	90 bp; 8	1883–1994	3/2	5/29	Genetic variation of extinct and extant populations	[76]
Hector's dolphin *Cephalorhynchus hectori*	206 bp	1870–1998	2/4	55/108	Loss of genetic diversity owing to fishery-related mortality	[20]
European otter *Lutra lutra*	9	1883–1993	3/3	67/58	Genetic consequences of population decline	[26]
Elephant seal *Mirounga angustirostris*	116 bp; 4	1500s–1990s	1/2	22/185	Effects of bottleneck on genetic diversity and on symmetry of bilateral characters	[19]
Grizzly bear *Ursus arctos*	8	1912–1999	1/3	110/136	N_e of Yellowstone grizzly	[31]
Common hamster *Cricetus cricetus*	240 bp; *DRB*[h]	1924–2000	2/3	20/31	Loss of MHC diversity in the *DRB* exon 2	[67]
Grey wolf *Canis lupus*	229 bp; 15+4[i]	1829–recent	4/2	33/22	Genetic variability and migration during population decline	[77]
	425 bp	1856–recent	-/2	32/399	Genetic variability and population size of extirpated US wolf populations	[23]
Arctic fox *Alopex lagopus*	292 bp; 5	1831–2004	2/2	21/41	Demographic bottleneck	[15]
Red fox *Vulpes vulpes*	354 bp	1911–2002	5/2	29/35	Genetic evidence for the persistence of Sierra Nevada red fox	[22]

[a]Within taxonomic groups, the order is by year of publication.
[b]Abbreviations: cpDNA, chloroplast DNA; MHC, major histocompatibility complex; RFLP, restricted fragment length polymorphisms.
[c]Unless otherwise noted, sequence length in base pairs (bp) stands for mitochondrial DNA and the single digit for the total number of microsatellites loci applied.
[d]The oldest and the most recent year of sampling.
[e]Number of populations and (/) number of temporal samples.
[f]Sample size of historical and (/) most recent samples.
[g]A microsatellite locus embedded in the MHC class I locus of brown trout.
[h]Gene that presents extracellular proteins to T lymphocytes.
[i]Refers to Y chromosome microsatellites.

Box 2. Tissue samples from natural history collections for genetic studies

Several types of tissue have been applied to obtain genetic data from NHC specimens. Here, we provide an overview of the most common tissue types used and their advantages and disadvantages in terms of potential damage to the specimens and the expected DNA quality.

Hides and skins
Epithelium tissue is a preferred source of DNA because damage is often insignificant and sampling is straightforward. Different preservation methods can, however, create substantial DNA degradation and PCR inhibition [48], which causes a high variance in DNA quality among samples. Superior DNA can be extracted from claws on hides of mammalian specimens [49] or from toe pads of birds [78].

Bones
DNA in bones is generally better protected than in epithelium tissue. However, DNA extraction is laborious because the material needs to be ground with a drill or a mortar. The use of maxilloturbinal bone material (i.e. thin bones inside the nasal cavities) might minimize damage to specimens [45], except where these bones are important for morphological analyses.

Teeth
DNA from teeth is generally well preserved given the hard tissue protecting it. Extraction protocols for teeth [51,77] are often identical to bones, although the efficiency can vary among different protocols [79]. To prevent the destruction of the whole tooth, only the root tip can be removed or the material following drilling inside the root cavity can be recovered. Nondestructive extraction methods have been developed [44,46].

Feathers
DNA has been extracted from the base of the feather calamus or the blood clot from the superior umbilicus. The latter is thought to yield higher amounts of DNA [80]. Careful selection of feathers results in little damage to the appearance of the specimen and removed feathers can be reattached [81].

Fish scales
Initially collected for use in age determination of individuals, several extraction protocols have been developed for scales [35,40]. Because they are plucked from fresh material, dermic and epidermic cells remain attached to the scale and dry up sufficiently fast to prevent DNA degradation [74].

Pinned insects
DNA can be extracted from pinned specimens using the whole insect or body parts [73]. Protocols conferring no external damage have recently been developed [82]. Different killing methods and storage conditions can affect the recovered DNA [83].

Fluid specimens
Many NHC specimens are stored in aqueous formalin. DNA extraction and PCR amplification from formalin-fixed specimens, however, is particularly difficult [50,54]. Formalin storage can cause frequent nucleotide misincorporations [50,55]. Particular care is needed to detect potential cross-contamination among samples because several specimens are often stored within the same fluid or because fluid among different storage containers could have been exchanged.

Herbarium specimens
Leaf tissue [34,84] and seeds [85] from plants dried and stored under controlled conditions and not preserved with chemicals can provide useful amounts of nuclear and chloroplast DNA [84].

Ethnographic artefacts
Animal and plant parts are also preserved in artefacts of ethnographic collections. Such collections can contain specimens that were collected before the major collection activities of NHC and thus might yield even longer time series (J. Groombridge, personal communication).

associated selective sweep has led to a significant loss of genetic variability [38]. This study illustrates the power of harnessing modern molecular genetic approaches with long time series data, either through the use of NHC samples or through long term studies [2].

Pitfalls and precautions

NHC hold an unchallenged wealth of specimens that reflect past and current biodiversity of our planet. However, molecular studies based on historical samples are challenging because genotype and sequence data obtained by PCR are often error prone. Consequently, precautions are needed to guarantee reliable genetic data.

Incomplete specimen records and small sample size

Unsurprisingly, most specimens of NHC collections were not assembled with the aim of carrying out genetic studies. As described by Fisher and Ford in their scarlet tiger moth study [37], this can considerably limit the utility of NHC collections for genetic studies. Collectors were likely to be biased in their sampling effort, particularly in relation to colour morphs and other phenotypic varieties. This might be less problematic when neutral genetic markers rather than functional genes are studied, but sampling localities, age and sex of the animals can still be biased. In addition, records for specimens from NHC are frequently incomplete, imprecise, missing or incorrect [39].

In a few instances, archival collections are reasonably comprehensive. For example, commercially and recreationally important fish species are often represented in systematic and continuous collections of scales or otolithes, sampled since the beginning of the 19th century to determine the age of individuals [40]. Few other taxa are represented by such comprehensive collections. Given that the number of historical specimens is limited, sample sizes can only be increased by searching longer for appropriate material, which is likely to exceed the time and effort spent on sampling modern material.

A related problem is that some specimens might not be suitable for molecular studies, because DNA needs to be physically extracted from tissue, which causes potential damage to the specimen (Box 2). Fifteen years ago, this problem led to a debate over the benefits and misuse of molecular studies based on NHC [41,42] and prompted several museums to establish guidelines for the use of specimens from their collections (Box 3). However, given the small amount of tissue now needed for extracting sufficient amounts of DNA for PCR amplification and the development of less or nondestructive DNA extraction methods [43–46], we believe that few NHC specimens should be out of bounds for molecular studies.

Molecular work with low-quality DNA

Numerous biological, physical and chemical factors affect the DNA quality of specimens from NHC. Most of these factors have been extensively described for ancient DNA work (reviewed in Refs [7,8]) and are also relevant for NHC, such as endogenous nuclease activity and hydrolytic damage. As a consequence, DNA extracted from historical material can be expected to be highly degraded and hence highly diluted similar to DNA derived from noninvasive

Box 3. Guidelines for genetic studies using natural history collections: study design

These guidelines are intended to outline the steps necessary to begin genetic investigations using specimens from NHC. They are divided into two sections covering the general (this Box) and the laboratory aspects of such projects (Box 4).

Collaboration with natural history museums
Unless specified otherwise by museums, researchers should provide a short project proposal that includes justification for the required material and evidence of the experience with such work. An agreement between the researchers and the museum is advantageous to clarify storage of and access to surplus extracted DNA and the future use of the genetic data. Museums should be regularly informed, acknowledged in publications and supplied with reprints of publications at the end of the study.

Sample selection
Records of specimens need to be verified to account for potential identification errors (e.g. imprecision or error in the location of a record [39]). Depending on the geographical and temporal scale of the intended study, specimens with imprecise records should be excluded. Only small tissue samples should be taken, causing the least amount of damage while providing a good likelihood of extracting sufficiently preserved DNA (Box 2).

Pilot study
A pilot study is invaluable to evaluate PCR amplification success and, where possible, to quantify the copy number of the target DNA. In addition, the frequency of genotyping errors in microsatellites and single base pair errors in sequence data should be assessed (Box 4). These results will clarify whether the scientific goals are realistic given the number of samples available, the sample quality and the molecular methods applied and will help to justify damage to additional specimens.

Sceptical attitude to own results
As records of specimens might be incorrect, results from genetic analyses should be evaluated carefully. Any samples for which specimen records and genetic data do not match or do not make biological sense should be treated with particular care. Moreover, results that might indicate methodological artefacts rather than biological findings have to be interpreted carefully.

Box 4. Guidelines for genetic studies using natural history collections: laboratory work

The main laboratory criteria relevant for working with specimens from NHC are based, in part, on those originally proposed for ancient DNA work [7,8]. However, there are some significant differences between ancient DNA and DNA from NHC (Box 1), which lead to the following guidelines.

Choosing appropriate genetic markers
Because DNA extracted from NHC specimens is highly degraded, amplicons of sequence data and microsatellite loci ideally should be <200 bp in size. All primers should be specifically designed for the species of interest to yield the best possible PCR efficiency and should have been extensively tested in modern samples. Species specific primers often can be redesigned within the flanking region of microsatellite loci and PCR multiplexing can be advantageous to delay the depletion of template DNA. The amplification of overlapping variable sequences of mitochondrial DNA will further diminish the chance that the sequence derives from a nuclear insertion.

Isolation of laboratory work area
Cross-contamination and contamination with exogenous DNA is a key concern when working with historical DNA. Therefore, DNA extraction and PCR preparation should be carried out in a dedicated and isolated laboratory with one way movement of DNA out of the laboratory. Only reagents exclusively purchased and maintained for working within such an environment must be used and extensive decontamination (e.g. UV radiation) of the laboratory, working surfaces and equipment is essential. Because the variation in the quantity of recovered DNA among NHC specimens can be very large, the number of simultaneously extracted samples needs to be kept small. The interspersion of samples that are likely to yield different haplotypes can further improve the detection of cross-contamination.

Negative control for extraction and PCR
Negative extraction and PCR controls need to be included to detect potential contamination in reagents and cross-contamination between samples.

Appropriate molecular behaviour
PCR amplification intensity of historical DNA is inversely related to product size, and products >500 bp are unusual. Deviations from such appropriate behaviour should be cause for careful checking and repetitions.

Reproducibility of genetic data
Several biological and nonbiological processes can damage DNA causing single nucleotide misincorporations [52,53]. Independent PCR and sequencing of unknown haplotypes are therefore required and particularly important sequences should be verified by cloning. Genotyping errors in microsatellites such as allelic dropout and false alleles are widespread in historical samples [15,51] and can be more common among loci with longer fragment size and among older samples [51]. Several independent PCR replications are required to attain a sufficiently high genotyping reliability [47]. Alternatively, the template DNA concentration can be estimated by quantitative PCR to adjust the number of PCR replications [56].

sampling [47]. In addition, different preservation methods can negatively affect the ability to extract, amplify and sequence DNA [48–50]. PCR amplification of historical DNA is, therefore, generally restricted to short amplicons (<200 bp) and is further vulnerable to contamination by recent DNA and PCR products from the study species. Because preservation methods can vary considerably, the variance expected in DNA quality among samples of similar age can be large and the risk of cross contamination is considerable. Consequently, sample age is not the only important factor that affects DNA quality ([44], but see Ref [51]). Molecular work with DNA from NHC specimens requires special precautions, including an isolated and dedicated laboratory environment (Box 4).

The cumulative damage to the DNA can also cause incorrect bases to be inserted during enzymatic amplification. The main source for these alterations are single nucleotide misincorporations ([7,52], and references therein). C to T transitions are the main type of such alterations that occur when an erroneous DNA strand is replicated during the first cycle of a PCR. Initially thought to be limited to ancient DNA, nucleotide misincorporations have recently been reported in studies based on specimens from NHC [15,52,53]. Estimates so far range from 17% to 21% of sequences that show one or more errors [15,53]. Special cases are formalin-preserved specimens, in which sequence alterations can occur at even higher frequencies [50,54,55]. Misincorporations might look like new alleles or new sequences and can therefore lead to systematic overestimation of the genetic diversity of past populations [53]. Consequently, repeated sequencing of independent PCR

products and cloning of important sequences are needed to ensure reliable genetic data [52].

The highly degraded and, therefore, diluted nature of DNA extracted from NHC specimens can cause a significant rate of genotyping errors when biparental genetic markers, such as microsatellites, are amplified by PCR. Two types of genotyping errors are likely: allelic dropout and false alleles. Allelic dropout is the stochastic nonamplification of one of the two alleles present at a heterozygote locus [47]. False alleles are PCR amplification artefacts that occur by the slippage of the taq polymerase during the first cycles of the PCR [47]. False alleles are less frequent than allelic dropouts. Because the rate of both types of error is inversely correlated with the concentration of the extracted DNA [47,51,56], a high frequency of allelic dropout can be expected in samples from NHC with low DNA quality. Consequently the genetic diversity measured will be systematically underestimated in historical samples compared with modern samples. This contrasts with the overestimation in sequence data that can occur owing to misincorporations and owing to false alleles. Several approaches have been proposed to achieve higher genotyping reliability for microsatellites in samples with low DNA quantity, including a predefined number of repeated and independent PCR amplifications [47] and the initial quantification of the template DNA concentration [51,56] by quantitative PCR assays.

Appropriate standards for reliable genetic data

On the one hand, the findings of molecular studies based on NHC can be misleading when ignoring the effects of sequence alterations and genotyping errors. On the other hand, it might be difficult to fulfil stringent standards, for example, when only a minute amount of DNA from a precious specimen is available and, as a consequence, only a small number of PCR reactions can be performed. Thus, although we propose guidelines for molecular genetic studies based on NHC samples (Boxes 3 and 4), we caution against sweeping, global standards. Instead, and similar to the approach advocated by Gilbert *et al.* [57] for assessing the authenticity of ancient DNA, we recommend a flexible approach, cognitive of the problems particular to every study.

Investigations using historical material need to be carefully assessed on a case-by-case basis by scrutinizing the magnitude of potential errors in historical genetic data in relation to the general findings of the investigation. For example, a minor and overlooked rate of allelic dropout might have a small effect on estimates of allele frequencies but could significantly alter the outcome of relatedness analyses with NHC samples. In particular, a sceptical attitude [7] towards ones own results is needed when findings can equally well be explained by methodological artefacts and biological processes.

Prospects

In the near future, advances in molecular technologies will enable access to more and more genetic information from specimens archived in NHC. This progress will allow us to shift from neutral genetic markers to specific genes under selection.

Gaining more genetic information from NHC samples

Mitochondrial sequences and microsatellites have been the preferred genetic markers in studies using NHC, which reflects the present genetic tools of choice for non-model organisms in ecology and evolution. Recently, single nucleotide polymorphisms (SNPs) have been put forward as an alternative to microsatellites [58]. SNPs have several advantages, including a known mutation model and a higher genotyping efficiency. Furthermore, they are suitable for highly degraded DNA because genotyping requires only short target DNA sequences (<100 bp) and protocols for genotyping of SNPs have been developed for degraded DNA [59]. However, the discovery of SNPs, that is, the selection of representative samples used for finding polymorphic sites, is crucial and can significantly bias the estimates of genetic variation within and between populations ([58], and references therein). To avoid this ascertainment bias, samples from different time periods and therefore also from historical samples could be used for the discovery of SNPs. Obviously, the degraded nature of historical DNA and the potential of nucleotide misincorporations will make this difficult. At present, the trade-off between these methodological constraints is unresolved and new molecular methods are needed to deploy the full potential of SNPs in NHC based studies. One such method is parallel pyrosequencing with a 454 instrument [60], which has been used in ancient DNA studies [61]. Overall, we believe that SNPs could soon become the marker of choice in studies based on NHC.

Genotyping and sequencing can rapidly deplete the precious and often limited amounts of DNA extracted from NHC specimens. This process can partly be delayed by multiplexing several loci within one PCR reaction, an approach now regularly used in microsatellite and SNP genotyping [59]. PCR multiplex amplification has also been used to obtain the complete sequence of the mitochondrial genome from the woolly mammoth (*Mammuthus primigenius*) from a 200 mg bone sample [62]. However, a complete and representative amplification of the limited DNA is preferred, which would provide a nearly unlimited copy number of genomic DNA for future molecular work. This could be achieved by applying whole-genome amplification (WGA) techniques. Several different WGA protocols have been developed to amplify the genomic DNA from minute amounts of template DNA, that is, from a few cells [63]. Most of these protocols rely on high quality template DNA, although protocols tolerant of degraded DNA have recently been developed [64]. We hope that in the near future novel WGA protocols will be adopted for use with NHC specimens.

Conservation genetics

DNA extracted from NHC specimens adds an important temporal dimension to the genetic study of endangered species and will remain pivotal in conservation genetics to assess phylogenetic positions and evolutionary significant (or management) units and to infer changes in population size and structure. Furthermore, contrasting past and recent genetic variability could serve as a retrospective approach of genetically monitoring populations for conservation and management [65]. Currently, there is growing

interest in understanding gene flow in a heterogeneous landscape by combining landscape and ecological data with individual based genetic data [66]. Historical samples might prove particularly valuable in this context because they enable direct tests of hypotheses concerning human-induced changes in gene flow patterns over time.

Beyond neutral markers

The main body of population genetic research using samples from NHC to date has focussed on temporal changes in genetic diversity of neutral genetic markers. By contrast, only a few NHC-based studies have identified allele frequencies of coding DNA sequences [38,67] or of microsatellites closely linked to sequences potentially under selection [68]. The relative ease with which one can now assay candidate genes and an increasingly large number of genetic markers makes it possible to study (natural) selection directly using temporal samples. Comparing the genetic variation from different time periods can be a powerful tool to detect molecular signatures of selection or environmental causes of selection. This has been exemplified by documenting insecticide resistance [38], evolutionary response to climate change [2] and human-induced allelic selection in maize [69].

Nevertheless, these approaches are not without caveats. Temporal variation of allele frequencies of a gene suspected to be under selection (or of a marker linked to it) occur because of selection, genetic drift or a combination of both. Consequently, statistical methods that separate the effects of selection from drift are required and these are often sensitive to assumptions about the demography of the populations [70]. Furthermore, different selection regimes can yield the same allele frequency dynamics [71], thus limiting the details that can be inferred from allele frequency dynamics data alone. Statistical analyses of selection are, however, a very active research field [70] and future methodological developments are likely to open up more possibilities.

Conclusions

Analyses of DNA from NHC samples have played an important role in conservation genetics by identifying processes that have shaped current levels of genetic diversity. A strong taxonomic bias is apparent among the studies to date (Table 1). Vertebrates and particularly fish predominate, whereas studies on plants and invertebrates are surprisingly rare. This bias might, in part, have arisen from biases in sample availability, conservation interests and methodological constraints, but the lack of more plant studies remains puzzling.

As NHC-based genetic work is coming of age, studies might fruitfully shift from investigating neutral genetic variation to studying the interplay of selection and drift. Although pioneered by Fisher and Ford 60 years ago [37], such direct studies of evolutionary change through the use of NHC samples are only beginning. Although technically demanding, such studies will be worth all the effort. Fisher and Ford [37] put it nicely: 'The spread of a gene in natural conditions is an event which repays detailed study since it provides an opportunity for examining evolution in progress. It has long been apparent to us that a careful watch should be kept for this occurrence and that whenever found it should, if possible, be analysed from two distinct points of view, ecological and genetic – a technique which has so far received much less attention than it deserves.' Combined with recent samples and, where possible, with ecological data that represent both current and historical time periods [39], NHC specimens provide a largely untapped resource for such investigations.

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